FISH TECHNOLOGY EXPERIMENT STATION
C.F.T.R.I.
MANGALORE.

VIBRIO FETUS INFECTION OF CATTLE

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

VIBRIO FETUS INFECTION OF CATTLE

With the Compliments of the
FAO INTERNATIONAL FOOD TECHNOLOGY TRAINING CENTRE
AT THE
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
MYSORE CITY
INDIA

VIBRIO FETUS INFECTION OF CATTLE

Revised edition

Edited by

J. A. LAING

Courtauld Professor of Animal Husbandry and Veterinary Hygiene,
Royal Veterinary College,
University of London

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
Rome 1960

First edition 1956

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Printed in Italy

CONTRIBUTORS

H. C. Adler	Department of Obstetrics and Gynaecology, The Royal Veterinary and Agricultural College, Bülowsvej 13, Copenhagen, Denmark
H. C. Bendixen	FAO Veterinary Consultant, Bülowsvej 15, Copenhagen, Denmark
E. Blom	State Serum Veterinary Laboratory, Bülowsvej 27, Copenhagen, Denmark
M. J. Christiansen	Veterinary Inspector, Henrik Pontoppidansgade 2, Aarhus, Denmark
W. Ross Cockrill	Chief, Section of Veterinary Medicine, Animal Health Branch, Animal Production and Health Division, FAO, Rome, Italy
E. Cohn	The Royal Veterinary and Agricultural College, Bülowsvej 13, Copenhagen, Denmark
Sir Thomas Dalling	Consultant, Animal Health Branch, Animal Production and Health Division, FAO, Rome, Italy
A. Florent	Chief of Section, State Veterinary Diagnostic and Research Laboratory, 99 Groeselenberg, Uccle-Brussels, Belgium
A.H. Frank	Animal Disease and Parasite Research Division, United States Department of Agriculture, Agricultural Research Service, Beltsville, Md., United States
S. L. Hignett	Wellcome Veterinary Research Station, Frant, Sussex, England

D. E. Hughes	New York State Veterinary College, Cornell University, Ithaca, New York
W. R. Kerr	Veterinary Research Division, Ministry of Agriculture for Northern Ireland, The Farm, Stormont, Belfast, Northern Ireland
N. Lagerlöf	Professor, Veterinary College, Department of Obstetrics and Gynaecology, Stockholm, Sweden
J. R. Lawson	Ministry of Agriculture, Veterinary Laboratory, New Haw, Weybridge, Surrey, England
E. Lindquist	Veterinary College, Oslo, Norway
D. J. McKinnon	Ministry of Agriculture, Veterinary Laboratory, New Haw, Weybridge, Surrey, England
F. Wöldike Nielsen	Director of Veterinary Services, Nyropsgade 37, Copenhagen, Denmark
N. O. Rasbech	Professor, Department of Obstetrics and Gynaecology, The Royal Veterinary and Agricultural College, Bülowsvej 13, Copenhagen, Denmark
G. Schmid	Professor, Institute for Veterinary Bacteriology and Parasitology, University of Berne, Switzerland
R. van der Sluis	Sanitary Services, Leeuwarden, Friesland, The Netherlands
Aa. Suurballe	Veterinary Inspector, Veterinär-Directoratet, Nyopsgade 37, Copenhagen, Denmark
J. I. Terpstra	State Serum Institute, Prof. Poelslaan 35, Rotterdam, The Netherlands
M. Tillmann	Professor, Free University, Department of Gynaecology, Berlin-Dahlem, Königin-Luisestrasse 49, Germany
M. Vandeplassche	Professor, Veterinary School, University of Ghent, Belgium
R. Wiidik	Institute for Veterinary Medicine, Stockholm, Sweden

The following papers were contributed to the meeting held in Copenhagen, 6-11 December 1954 :

A. General consideration of infectious diseases and their relationship to bovine infertility

Importance of infectious diseases with special reference to influence on fertility in cattle, by H. C. Bendixen

Clinical and epidemiological aspects of mating diseases of cattle in the Netherlands, by L. van der Sluis

Trichomonas infection in cattle, by M. Vandeplassche and F. Paredis

Pleuro-pneumonia-like organisms in Danish bulls, by B. E. Albertsen

Preliminary report of a serological survey of leptospirosis in aborting cattle in Denmark, by K. L. Fennestad and C. Borg-Petersen

Comparison of modes of transmission of infection, by J. A. Laing

B. Vibriosis in cattle: clinical aspects, including therapeutic and prophylactic treatment

Vibrio fetus infection in Sweden, by N. Lagerlöf and H. Boyd

Vibriosis in cattle, by S. L. Hignett

The occurrence of vibriosis in cattle in Norway: prophylaxis and treatment, by O. Garm

Continued investigation into *Vibrio fetus* as a cause of infertility in cattle, by H. C. Adler, B. E. Albertsen and L. Szabo

The symptoms of bovine vibriosis, by J. R. Lawson

Experimental treatment of *Vibrio fetus* infection, by J. R. Lawson

Treatment of *Vibrio fetus* infection in bulls, by M. Vandeplassche, F. Paredis and A. Florent

Treatment of *Vibrio fetus* infected bulls, by H. C. Adler

Treatment of *Vibrio fetus*-infected female animals, by H. C. Adler

The passage of antibodies into the uterus and vagina, by W. R. Kerr

A field observation on vibriosis in cattle, by G. Schmid

C. Vibriosis in cattle: diagnosis

Laboratory techniques in the diagnosis of *Vibrio fetus* infection' by D. J. McKinnon

Preparation of *Vibrio fetus* antigens for serological testing, by J. I. Terpstra

Laboratory methods in the diagnosis of vibriosis in cattle and their results, by A. Florent

Notes on vibriosis with special reference to the isolation of *Vibrio fetus* from semen and preputial fluid, by D. E. Hughes

Investigations into the antigenic structure of *Vibrio fetus* isolated from cattle, R. W. Wiidik and G. Hlidar

Method for diagnosing *Vibrio fetus* infection in the bull, by H. C. Adler

Comparative serological examinations of 21 bovine strains of *Vibrio fetus*, by H. Juhler

The diagnosis of *Vibrio fetus* infection by means of the mucus agglutination test, by E. C. Cohn

Observations on the incidence, diagnosis and transmission of vibriosis in cattle and the identification of *Vibrio fetus*, by A. H. Frank and J. B. Bryner

Vaginal and uterine antibodies in the bovine with particular reference to *Brucella abortus*, by W. R. Kerr

D. The Handling of semen for artificial insemination

Problems related to the hygienic quality of bull semen as it is used in artificial insemination, by E. Blom

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INTRODUCTION

The first edition of *Vibrio Fetus Infection in Cattle*, edited by J. A. Laing, was published by the Food and Agriculture Organization of the United Nations (FAO) in 1956. Its publication followed meetings convened by FAO and attended by well-known specialists on this disease. The papers presented at these meetings were combined in an effort to give a comprehensive idea of current views on *Vibrio fetus* and the disease it produces in cattle. Complete sections of some of the papers, rearrangement of others, and discussions at the meetings provided the bulk of the material for the publication; in addition, some factual matter was added to aid continuity and to provide background material.

In 1955 FAO established a panel of experts on vibriosis. Meetings of the panel were held in 1956 and 1959 respectively, at which progress in the study of the disease was considered. Sufficient new information was presented to make it advisable to produce a second edition of the publication in order to bring the original publication up to date.

A considerable part of this second edition, especially that on the detailed descriptions on diagnostic techniques and the preparations of media are unchanged. In other parts, somewhat extensive alterations have been made to incorporate the results of recent research and observations.

It is gratifying to find that in some countries in which the measures outlined in the first edition have been adopted, the disease has been brought under control and may soon be so reduced in incidence that it no longer constitutes a problem in cattle breeding.

On the other hand, in some parts of the world, bovine genital vibriosis continues to be an important limiting factor in cattle breeding

and its prevalence is being increasingly recognized as improved diagnostic techniques are employed.

Some of the most recent research work indicates that an explanation may now be available for the different types of *Vibrio fetus* which cause true venereal enzootic abortion and sporadic abortions in cattle.

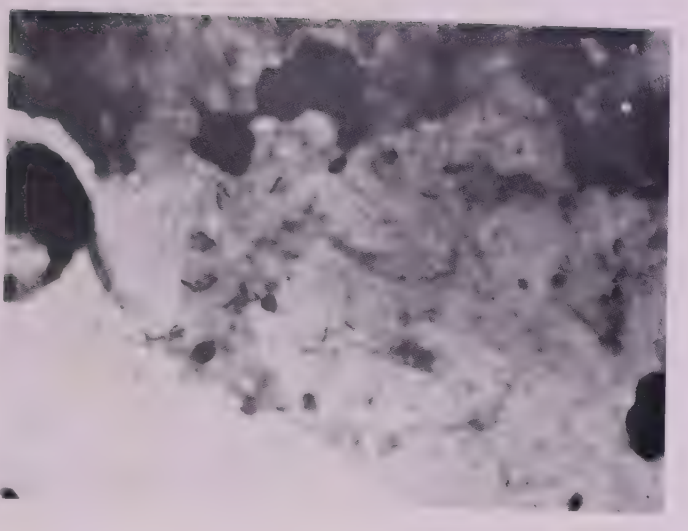

FIGURE 1. – Vibrio fetus *in foetal stomach contents.*
Gram × *1,200.*

FIGURE 2. – Vibrio fetus *in culture. Note long forms.*
Gram × *1,200.*

Reproduced from *Fertility and Infertility in the Domestic Animals* (J. A. Laing), by courtesy of Ballière, Tindall and Cox, London.

I. VIBRIO FETUS

Vibrio fetus is a gram-negative *vibrio* found in material from naturally-infected animals and in fresh cultures in short S-shaped and, less frequently, in slightly more elongated forms. Very long spiral forms are often found in old cultures. In length and width this organism varies from 1.5 – 5.0 μ and 0.2 – 0.3 μ (Figures 1 and 2). Usually it is motile, but non-motile strains occur naturally and motile strains may become non-motile on subculture.

Most strains are micro-aerophilic, although some will grow aerobically when adapted to laboratory conditions. For primary isolation, media containing blood or serum are an advantage. Once the organism becomes established, such enriched media can often be dispensed with and cultures maintained on other media such as thiol. Recently, semi-solid media have been extensively used, although they are less useful for obtaining primary cultures from contaminated material, as they do not allow separation of *V. fetus* from other organisms because of the slow growth of *V. fetus*, which may be quickly overgrown by contaminants even on solid media.

Although *V. fetus* has been known and associated with disease in cattle and sheep for many years it has until recently been recognized only on its morphology and, to some extent, on its oxygen requirements for growth, although it was appreciated that it might be only one member of a large group of morphologically similar but otherwise diverse organisms.

It has been shown that *V. fetus* can be differentiated from other *vibrios* by differential cultural tests (Bryner and Frank, 1955 a and b). More recently there has been evidence that, on the basis of its biochemical reactions, its predilection sites and its pathogenicity, *V. fetus* can be divided into two types: the first, the natural pathogen of the bovine prepuce and genital tract causing infertility and occasionally

abortion; the second, which has as its natural habitat the intestine of cattle, sheep and pigs, and sometimes invades the pregnant uterus of the sheep and cow, thereby causing abortion (Florent, 1959). It has been suggested that these two types should be named *V. fetus* (*venerealis*) and *V. fetus* (*intestinalis*). This nomenclature has been followed throughout this monograph. These two types correspond to Types I and III of Terpstra (1956).

Differential cultural tests

Experiments with these tests have shown that organisms with the general cultural characters described above and with morphology considered to be that of *V. fetus* can be divided into two main groups. The first, which are catalase-positive with a catalase number of 30 or more, may or may not produce hydrogen sulphide according to their type and the medium on which they are grown, and have been isolated only from aborted foetuses. Similar organisms have also been isolated from the male and female reproductive tracts of infertile animals. Organisms of this type are considered to be true *V. fetus*.

Organisms of similar morphology and general cultural characters, but which are catalase-negative and produce large quantities of hydrogen sulphide, have not been isolated from aborted foetuses. Although they may be found either together with the catalase-positive type or alone in the reproductive tracts of infertile animals, they are also present in fertile cows and bulls, including bulls which have never bred, and are thought not to be associated with infertility. They are considered not to be *V. fetus* (Tables 1 and 2). *Vibrio coli* is catalase-positive but produces hydrogen sulphide.

Table 1. – Differentiation of *vibrios* isolated from aborted foetuses and reproductive tracts of cattle

Source of *vibrio*	Number of cultures	Catalase production		Differential tests hydrogen sulphide production		Deep stab	
		positive	negative	positive	negative	growth	no growth
Aborted foetuses	27	27	0	0	27	0	27
Vaginal mucus	76	64	12	12	64	12	64
Female reproductive tract, post-mortem . .	4	4	0	0	4	0	4
Bull semen	44	21	23	23	21	23	21
Bull prepuce	11	2	9	9	2	9	2
Bull prepuce post-mortem	2	1	1	1	1	1	1

Table 2. – Types of *vibrio* isolated from herds of cattle with impaired fertility

Herd examinations	*Vibrio* status of herds			Types of *vibrio* isolated		
	number of herds	infected	not infected	catalase-positive	catalase-negative	both types
Single collection .	26	17	9	13	4	0
Two or more collections	32	20	12	5	2	13
Total . . .	58	37	21	18	6	13
Percentage	100	64	36	49	16	35
				100		

Catalase activity

A modification of the catalase test, as described by Blom and Christensen (1947), is used to determine the catalase activity of *vibrio*. Three-day growth of *vibrio* in thiol media is used for the test. The amount of growth is first recorded, then a volume of 3 percent hydrogen peroxide solution (prepared by adding 1 part of commercial 30 percent H_2O_2 to 9 parts of distilled H_2O) equal to the amount of medium is added to the culture. The cotton wool plug is replaced by a special stopper provided with a capillary tube. The culture tube is immediately inverted and the level of the contents marked with a wax pencil. The contents of the tube are then further mixed by inverting the tube a few times, after which it is left inverted, placed in a wire basket over the sink and allowed to stand for 20 minutes. The volume of liquid displaced by gas is measured in millimetres and recorded as the catalase number. Cultures displacing 5 millimetres or less of liquid are considered to be catalase-negative and those displacing more than this amount are regarded as catalase-positive.

Production of hydrogen sulphide

The culture medium for this test is nutrient broth composed of peptone 10 grammes, sodium chloride 5 grammes, and beef infusion to make 1,000 millilitres. This medium is inoculated with 0.5 millilitre of a concentrated three-day growth of *vibrio* from thiol medium, after which a strip of filter paper impregnated with lead acetate is inserted into the tube and held in place by the cotton plug. The culture is

then incubated in an atmosphere of 10 percent carbon dioxide at 37.5°C. for 24 hours. An appreciable darkening of the lead acetate strips in 24 hours is considered to denote a positive hydrogen sulphide reaction; slight or no darkening is considered to be negative.

Differentiation of two types of *Vibrio fetus*

Vibrio fetus can be differentiated into the two types, first, by the fact that *V. fetus* (*venerealis*) grows more rapidly and luxuriantly in an atmosphere containing 10 percent carbon dioxide in which the oxygen has been reduced to one third of normal by the introduction of nitrogen; second, that it produces no hydrogen sulphide when grown in semi-fluid Martin broth containing 0.02 percent cystine; and third, that it does not grow in medium containing 1 percent glycine. *V. fetus* (*intestinalis*) on the other hand, has the contrary reactions in Martin broth with cystine and in glycine, and grows in an atmosphere of 10 percent carbon dioxide without further reduction in oxygen tension.

The reactions and characteristics by which *V. fetus* may be differentiated from other *vibrios*, and by which *V. fetus* (*venerealis*) and *V. fetus* (*intestinalis*) may be distinguished, are set out in Table 3.

Antigenic structure

It has been known since the early work of Theobald Smith and his co-workers that the organism recognized as *V. fetus* is made up of a group of strains of some antigenic complexity (Smith, 1918; Smith and Taylor, 1919; Smith, Little and Taylor, 1920; Smith, 1923; Smith and Orcutt, 1927). Two main antigenic groups exist: thermolabile "H" or flagellar antigens and thermostable "O" or somatic antigens. The former produce a flocculent reaction on agglutination and the latter a granular reaction, although the difference between the two types of agglutination is not so distinct as with some other motile organisms, particularly the *Salmonellae*. In addition, a capsular or "K" antigen is present (Wiidik and Hlidar, 1955).

Recent investigations on the antigenic structure of the organism are in some ways contradictory and difficult to interpret in relation to one another, largely because of the varying techniques employed by different workers. Publications by Ristic, White, Doty, Hertzberg

Table 3. – Distinction of the two types of *Vibrio fetus* and their distinction from other catalase-positive animal *vibrios*

	V. fetus	
	I *venerealis*	II *intestinalis*
Growth atmosphere		
(I) 10 % CO_2	±	+
(II) 10 % CO_2 with O_2 tension reduced to one third	+	+
Aerobic growth	–	–
Anaerobic growth	–	–
Catalase reaction	+	+
H_2S production		
(I) In semi-fluid Martin broth	–	–
(II) In semi-fluid Martin broth with 0.02 % cystine	–	+
Growth in medium containing 3.5 % NaCl	–	–
Growth in medium containing 1 % glycine	–	+
Predilection site	Prepuce of bull; genital tract of cow and heifer	Intestine
Infected or carrier species	Cattle	Cattle Sheep Pigs
Ability to survive in the bovine vagina	+	–
Pathogenicity in relation to fertility	Infertility, and occasionally abortion in cattle	Abortion in cattle and sheep

and Saunders (1957), Ristic, White and Doty (1958), Liess (1958), Mitscherlich and Liess (1958) and by Morgan (1959) may be consulted. It is necessary to know more concerning the antigenic relationship between *V. fetus* and other *vibrios*, the relationship between different strains of *V. fetus*, and also whether *V. fetus* (*venerealis*) and *V. fetus* (*intestinalis*) can be differentiated serologically.

In addition to the antigenic differences and relationships between various strains of *V. fetus*, the antigenic relationship of this organism with others must be considered. Because of the similar form of the disease they cause, and because agglutination testing is used in the diagnosis of both, comparison with *Trichomonas foetus* is particularly important. It has been shown that most mucus samples from animals possessing agglutinins to *T. foetus* will agglutinate *V. fetus* aspecifically. On the other hand, agglutinins developed against *V. fetus* do not react with *T. foetus*. It is believed that, although these organisms possess common antigens, they are lost by *T. foetus*, *in vitro*. This is thought to be so because *V. fetus* is not agglutinated by trichomonas-immune antisera produced in rabbits and because common agglutinins in the mucus of cows infected with *T. foetus* cannot be absorbed with suspensions of trichomonads.

II. EPIDEMIOLOGY

In considering the epidemiology of genital vibriosis in cattle it is necessary to differentiate between the effects of the two strains, *V. fetus* (*venerealis*) and *V. fetus* (*intestinalis*). The evidence which has accumulated, at first from Denmark and later from several other countries (Anon. 1958), from the experimental exposure of heifers for diagnostic purposes, indicates that most mature females not previously exposed are susceptible to infection with the venereal strain. There is, however, some earlier evidence which indicates that immature females may be somewhat refractory. The bull is susceptible to infection, but attempts to produce experimental infection have met with varying degrees of success and it seems likely that all bulls are not equally susceptible. Experimental infections sometimes tend to resolve spontaneously in a short time (A. H. Frank, personal communication). Genital vibriosis occurs also in sheep but is almost certainly caused solely by *V. fetus* (*intestinalis*) and, if cross-infection between sheep and cattle occurs, it is fairly certain that it is only with this strain and that the disease in sheep is therefore not epidemiologically important with regard to cattle, except in relation to those sporadic abortions caused by *V. fetus* (*intestinalis*). Infection with *V. fetus* (*venerealis*) has not been recorded as a natural disease in species other than cattle and considerable difficulty has been experienced in infecting other animals experimentally. *Vibrio fetus* (*intestinalis*), however, has been found in the intestine of pigs as well as in the genital and intestinal tracts of cattle and sheep (Florent, 1959).

Infection with *V. fetus* (*venerealis*) is almost invariably venereal, and in fact infection by other routes is probably so rare, except between bulls used for artificial insemination, that it can be discounted as an epidemiological factor. Both the cow and the bull may, therefore, spread the disease, the latter probably being the more important. Where

communal bulls are used, or where mature bulls are hired or traded in, the spread of infection by such animals from herd to herd can often be easily traced. Carrier females, in herds where the disease has become chronic and the majority of females immune to infection do, however, play an important part in spreading the disease. Infection can be spread by artificial insemination both in semen stored at 5°C. and at −79°C. and, where no system of diagnostic tests has been applied to bulls before their use for artificial breeding, widespread dissemination of the disease has been known to occur. Infection may also be spread from bull to bull at artificial insemination centres, probably most often through contact with other animals, instruments, etc., used in the process of collecting semen and sometimes via infected bedding. These modes of infection must always be borne in mind under the special conditions obtaining at artificial insemination centres.

Once infection is established in the bull it remains for long periods and may be permanent, although it is possible that spontaneous recovery may sometimes occur. There are records of infection persisting for periods up to six years. The disease is to some extent self-limiting in cows in most herds after a period of three to six months. It is generally believed that this occurs in association with the development of some immunity to the infection. This modifies the form and course of the latter part of the disease, so that the majority of females begin to breed normally. How long this immunity lasts and how effective is immunity developed against one strain in protecting animals from infection with antigenically different strains is unknown. Recent observations indicate that a single exposure to infection does not provide a level of immunity adequate to prevent subsequent reinfection (Melrose, Morgan and Stewart, 1959). So far as one may deduce from the development of detectable specific mucus agglutinins, acquisition of immunity varies considerably. In one series of observations the time of the first appearance of agglutinins varied from 14 to 105 days after exposure, the average being 42 days. Sometimes agglutinins may persist for up to eight years. It is extremely doubtful, however, if the persistence of agglutinins necessarily indicates the development of effective immunity; it may equally well be associated with the " carrier " state. " Carriers " do occur, as has been mentioned above and, even if they are infrequent, can be an important factor in maintaining the disease within a herd and spreading it to others.

Where the disease is being transmitted from artificial insemination centres, its effects can be modified by the use of antibiotics in semen. Normal fertility can be obtained in this way but there is no doubt that on some occasions the organism remains viable in semen so treated and infection is transmitted. The effectiveness of antibiotic treatment of semen probably depends on the technique used, and slight variation from the successful techniques may allow viable organisms to remain and to be inseminated. A false sense of security may thus be produced.

Vibrio fetus (*intestinalis*) would appear to be a sporadic pathogen which is not spread by the bull, infection of the uterus occurring most probably from the bowel under conditions which are not yet understood. The possibility that sheep and pigs may act as reservoirs of infection must be borne in mind.

The spread of *V. fetus* (*venerealis*) infection through normal trade in mature breeding stock and in many cases by means of artificial insemination, together with the earlier lack of appreciation of the mode of transmission of vibriosis and the serious effects of the disease, have combined to produce a high incidence in many countries. In most cases it is not possible to form a precise estimate of the true incidence, partly because of the varying effectiveness of the diagnostic measures used and partly because in most countries the disease is not officially notifiable. It has been diagnosed in 28 countries, but in many others, while its presence is not suspected or is believed to be absent, diagnostic facilities are such that it may have been overlooked. In 1949/50, in one country, 4.6 percent of aborted foetuses examined were infected; by 1952/53, with the introduction of control measures, the proportion had fallen to 2.8 percent, although more foetuses had

Table 4. – Incidence of *Vibrio fetus* infection

	1949/50	1952/53
Number of foetuses examined	1 088	1 717
Number infected with *V. fetus*	50	48
Number of samples of vaginal mucus examined for agglutinins	1 692	2 470
Number positive	286	325
Number of outbreaks of infertility investigated	343	338
Number due to *V. fetus* infection	97	79

Table 5. – Incidence of *Vibrio fetus* infection

Outbreaks of infertility investigated in five-year period	191
Number caused by *V. fetus*	59
Number of placentae examined at full term in five-year period	223
Number infected with *V. fetus*	59
Number of aborted foetuses examined in five-year period	47
Number infected with *V. fetus*	6
Number of specimens of vaginal mucus examined in five-year period	1 043
Number infected with *V. fetus*	8
Number of samples of semen and preputial washings examined in five-year period	908
Number infected with *V. fetus*	3

Table 6. – Frequency of infection in 76 artificial insemination centres at first examination (Adler and Lindegaard, 1958)

	Number of centres	Number of bulls	Number of infected bulls	Infected bulls
				Percentage
V. fetus + centres	32	515	140	27.2
V. fetus – centres	44	346	0	0
Total	76	861	140	16.3

been examined (Table 4). In another country, the incidence in aborted foetuses fell from 33 percent in 1950/51 to 13 percent in 1952, after the disease had been made notifiable. The disease was similarly widespread elsewhere (Table 5) and in some countries a high proportion of the bulls used for artificial insemination were infected (Table 6; see also Morgan, 1956).

In recent years the incidence has fallen in many other countries with the introduction of control measures. In the United Kingdom, for example, isolations of the organism from aborted foetuses reached a peak in 1951 when 11.6 percent of specimens were positive. Since then there has been a steady decrease in the number of isolations until in 1958 only 1.3 percent (3 of 237) foetuses were positive (J. R. Lawson, personal communication). There has been a comparable decrease in the number of samples positive to the mucus agglutination test.

III. SYMPTOMS

Infection with *V. fetus* (*venerealis*)

The common seat of infection in the bull is considered to be the preputial mucosa, but no clinical abnormalities can be observed in animals which are carriers or transmitters of the organism. The semen is normal.

In the cow and heifer, infection is at first acute and gradually assumes a chronic form. Infertility is usually associated with the acute, and abortion with the chronic phase, but abortion may occur also in the period of acute infection. The change from the acute to the chronic disease may be associated with the development of resistance to infection, but the exact relationship between the two forms of the disease is not yet clear.

At the onset of the disease, vaginal examination often shows a catarrhal vaginitis resulting in increased mucus secretion. This may last for three to four months. The mucus is generally clear but may be slightly cloudy or, exceptionally, purulent. The vaginal mucosa may be reddened, particularly in the region of the cervix, which may also be swollen and reddened because of the development of catarrhal cervicitis. Endometritis also develops at the same time but may not always be clinically detectable.

During the acute stage of the infection fertility varies considerably, about 15-45 percent of animals becoming pregnant to the first mating, the remainder returning to service, often repeatedly.

The level of fertility in the acute stage of infection has been demonstrated by observations made on heifers infected for experimental purposes (J. R. Lawson and D. J. MacKinnon, personal communication) (Tables 7 and 8).

Usually the inter-service periods are irregular and abnormally long. There is evidence which suggests that conception may actually occur

Table 7. – Fertility in *V. fetus* (*venerealis*) infection

	Number of heifers	Number of heifers pregnant	Total number of services or inseminations	Number of services or inseminations per pregnancy
Infected heifers	101	82	526	5.2
Heifers not exposed to infection failed to become infected or were cured by treatment	108	107	206	1.9

Table 8. – Comparison of numbers of services required for pregnancy in non-infected and infected heifers

Service or insemination following which pregnancy occurred	Infected heifers	Non-infected heifers
1	5	54
2	8	32
3	10	11
4	12	3
5	17	3
6	12	2
7	6	2
8	6	0
9	1	0
10	2	0
11	1	0
12	2	0
	82	107

in many cases but be followed by foetal death and reabsorption or expulsion of the foetus. The exact mode of pathogenesis in vibriosis in all of its various stages is not, however, definitely known. If mating is continued, most animals become pregnant after about three months but a small number may fail persistently to become normally pregnant.

Later, the disease now being chronic, fertility becomes fairly satisfactory. Only maiden heifers and those cows which have not pre-

viously been exposed to infection become infertile because they contract the acute disease. Difficulties are usually experienced annually with each succeeding group of heifers.

Some pregnant animals may abort, very often those which have become pregnant to the first service. Abortions have been recorded at all periods of gestation but are most common about the fifth and sixth month. The abortion rate is usually low.

Infection with *V. fetus* (*intestinalis*)

So far as is known, the only symptom related to fertility is the occurrence of sporadic abortions, usually, but not invariably, in the latter half of pregnancy.

IV. DIAGNOSIS

Although the symptoms described above may lead to the suspected presence of *V. fetus* infection in a herd, a definite diagnosis of the disease cannot be made on them alone. They resemble closely the symptoms shown in trichomoniasis, in other infectious diseases and also in some forms of non-infectious disease of the reproductive organs. Therefore, once the disease is suspected on clinical grounds, steps must be taken to differentiate it from these other conditions, always bearing in mind that other diseases causing infertility may co-exist with genital vibriosis. Accurate diagnosis can be made either by isolating the organism from material from suspected animals or by means of specific serological tests. Bacteriological diagnosis is applicable to both males and females. Serological methods can be used directly on females only, although they may be employed indirectly for the diagnosis of the disease in bulls, after test-mating of heifers.

While the history and symptoms of the disease are not in themselves diagnostic, they are of considerable value in selecting animals from which it is most profitable to take material for diagnostic tests, should it not be possible to test all the suspected animals. Further, they do indicate to some extent which animals, and particularly which bulls, may be infected. The diagnostic methods employed depend to a great extent on local conditions. Very often an initial definitive diagnosis is made from the bacteriological examination of an aborted foetus, but on many occasions where an investigation is begun because of symptoms of infertility, it may be convenient to use the mucus agglutination test as a screening test, and on others it may be best to attempt diagnosis by the immediate examination of bulls.

The cow and heifer

Bacteriological examination of suspect material

The materials which may be examined bacteriologically are aborted foetuses and their placentae, and mucus or muco-purulent material from the vagina.

Foetal and placental tissue should be as fresh as possible when examined. Portions of lung, spleen and the contents of the rumen and abomasum are removed with sterile precautions and separately ground up with sterile sand and saline. Where the foetus is grossly contaminated, or when, as sometimes occurs, the carcass has been eviscerated by dogs or vermin, the brain may be used in a similar way. The resulting suspensions are inoculated separately into suitable media (see below). In the case of very small foetuses, cultures are made from amniotic fluid and any oedematous areas of the placenta. Such areas may be readily sampled by searing the surface to dry it, reflecting the seared surface, and taking up loopfuls of the underlying oedematous tissues.

Smears from the ruminal and abomasal contents are stained by Gram's method and examined microscopically. The contents of these organs are also examined under dark-ground illumination for motility.

Cultures are incubated at 37°C. in an atmosphere in which the oxygen tension has been reduced to one third by the introduction of nitrogen, and to which 10 percent of carbon dioxide has been added, for three days. They are then examined and, if the results are negative, re-incubated for a further three days before the final examination.

After incubation, the culture plates are examined with a low-power dissecting microscope for suspicious colonies. Considerable variation in colonial type occurs, but what might be considered the basic types are shiny, pale-gray, semi-translucent, flattened, conical colonies, small and large ones often appearing on the same plate. Individual colonies, however, do not breed true. Large or small colonies appear on subculture. Sometimes the growth appears as if several colonies had coalesced into a large mucoid mass, but at other times the only evidence of growth is a slight "frosting" of the surface of the medium. This variation in colonial character makes extensive microscopic examination important. All suspicious colonies are smeared and, if there are none, a general smear is made from the plate. Smears are stained by Gram's method. Slopes are examined as far as possible in the same

way, particular attention being paid to the fluid at the bottom of the tube. Identification of *V. fetus* is aided by the use of the phase-contrast microscope with wet films.

V. fetus is Gram-negative and usually actively motile. In smears made from the stomach contents it may appear in the "comma" form, but the forms most commonly seen are those which resemble the artist's flying seagull. In culture on solid medium, these forms appear but longer organisms with several convolutions are more frequently found (Figures 1 and 2).

Once isolated, the organisms must be differentiated from other *vibrios*, and the two types *V. fetus* (*venerealis*) and *V. fetus* (*intestinalis*) from one another, by the tests described earlier.

In the period 1947-54, in one laboratory 2,727 foetuses were examined and, from 196, *V. fetus* was isolated. Of these, 130 were positive both on culture and direct smear of the stomach contents, 57 were positive on culture only, and 9 positive on smear only.

Vaginal mucus for bacteriological examination should be collected at oestrus, as specimens taken from infected animals at other times of the oestrous cycle are less likely to prove positive. In one experiment 82 percent of samples cultured at oestrus proved positive, whereas only 28 percent of those taken at other times between primary infection and the establishment of pregnancy were positive. Mucus taken during the period between 7 and 21 days after first exposure to infection is also likely to be positive. This is made use of in the diagnosis of infection in bulls, following test-mating of maiden heifers (see below).

The mucus must be sent for examination as rapidly as possible after collection. It is inoculated into the same type of media as are used for the cultivation of foetal and placental tissue and the media is examined after similar incubation. It is considered important that, in the inoculation of plate cultures, some mucus should be left on the plate and not simply smeared over the surface and removed.

Serological examination of suspect material

The serum-agglutination test

It is generally considered that the serum-agglutination test is of very limited value for diagnostic purposes and that it is much inferior to the mucus-agglutination test. The serum-agglutination titres

of infected animals are not regularly increased by the stage of the infection, except possibly just after abortion, whereas the mucus titre is much more regularly increased whether or not abortion occurs and, after abortion, very high levels may be reached. Recent investigations indicate that the complement fixation test may be of value for diagnostic purposes but more information is necessary before it can be applied as a routine.

The mucus-agglutination test

This test, which is a modification of the test devised by Pierce (1946, 1947) and by Florent and Willems (1947) for the diagnosis of *T. foetus* infection, is carried out on vaginal mucus. Because there is delay between exposure to infection and the development of agglutinins in vaginal mucus, and because the titre tends to fall from its maximum with time, it is necessary to select the animals from which specimens shall be taken, if all suspected cows and heifers cannot be sampled.

Table 9. – Time of appearance of agglutinins in the vaginal mucus [1]

Number of days after exposure	Number of heifers		Total
	naturally infected	artificially infected	
0–14	1	2	3
15–28	2	8	10
29–42	10	14	24
43–56	11	14	25
57–70	8	4	12
71–84	3	5	8
85–98	4	2	6
99–112	3	0	3
113–126	2	0	2
127–140	1	0	1
141–154	1	0	1
	46	49	95

[1] Specimens taken for testing every seven days.

Table 10. – Duration of period of reaction to the mucus-agglutination test in animals observed for at least two months after disappearance of reaction

Duration of reaction (months)	Number of animals		Total
	naturally infected	artificially infected	
2½	1	1	2
3	0	6	6
4	2	1	3
4½	2	0	2
5½	1	0	1
6	1	0	1
6½	1	1	2
8	1	0	1
9	1	0	1
9½	3	0	3
11½	1	0	1
12	1	1	2
14	0	1	1
15	0	1	1
	15	12	27
(years) 5½	3	1	4

Very few animals which have never been exposed to infection show agglutinations at what would be regarded as a positive level. In one series of observations only two of 600 maiden heifers gave suspicious reactions (J. R. Lawson and D. J. MacKinnon, personal communication). In another survey, only 0.9 percent of animals gave reactions which could not be associated with infection (Boyd and Reid, 1960). Agglutinations appear at varying periods after first exposure.

The results obtained in one series of observations in which mucus samples were tested at seven-day intervals, after primary exposure to infection (J. R. Lawson and D. J. MacKinnon, personal communication), showed that a few animals reacted within 14 days and some not until more than 20 weeks afterward but the majority were positive between four and ten weeks after exposure (Table 9). Further observa-

tions on these animals showed that the reaction persisted for periods varying from two and a half to sixteen and a half months (Tables 10 and 11) and in a few cases for as long as five and a half years but that, in the case of a few animals, the reaction fluctuated between positive and negative (Table 12). In many animals positive mucus titres remain after the infection has resolved; in some few there may be no development of agglutinins, although this is probably very rare.

Table 11. – Duration of period of reaction to the mucus agglutination in animals disposed of while reaction was still present

Duration of reaction (months)	Number of animals		Total
	naturally infected	artificially infected	
1	2	0	2
2	1	0	1
4	1	0	1
5	2	8	10
6	3	7	10
7	4	7	11
8	0	1	1
9	0	1	1
11	0	1	1
13	1	0	1
14	1	1	2
15	2	3	5
16	2	2	4
16½	1	0	1
	20	31	51

Specimens taken during oestrus usually give negative results, although the same animals may be positive at other times in the oestrous cycle. Mucus taken in one experiment from infected animals at oestrus was positive in only 18 percent of cases, whereas 68 percent of samples taken at other times in the oestrus cycle contained agglutinins.

In herds where the disease has become chronic among the adult cows and where, because a separate and uninfected bull is used on heifers, these are not exposed to infection, samples may most profitably

Table 12. – Fluctuating agglutination reactions

History	Number of animals under observation	
	naturally infected	artificially infected
Reactions for two months: then negative for two months: then reactions for two months	—	2
Reactions for three months: then low or negative readings for two months: then reactions for three months	—	3
Reactions for five months: then low or negative readings for two months: then marked reactions for seven months	1	—
Reactions for five months: then negative for one month: then low or negative readings for nine and one half months	1	—
Reactions for five and a half months: then negative for two months: then low readings for five and three quarter months	1	—
Reactions for seven months: then negative for one month: then low readings for seven and a half months	1	—
Reactions for seven months: then negative for one month: then low readings for four months: then negative for three months	1	—
Reactions for seven and a half months: then negative or low readings for five and a half months: then negative for three months	1	—
Reactions for eight months: then negative for one and a half months: then marked reactions for six months	1	—
Reactions for eleven and a half months: then negative or low readings for three and three quarter months: then negative for two months	—	1
	7	6

be taken from cows which have been bred after having their first calves. Generally speaking, animals with old chronic infections are less suitable subjects for testing because the agglutinin titre has be-

come reduced and samples are best sought among animals in which the disease is still in its acute stage. Where it is desirable to know the condition of a whole herd, and particularly where it is wished to follow the course of the disease over an extended period, samples should be taken from all females over one year old. When mucus is contaminated with blood, false positive results may be obtained.

Because of the varying mucus agglutinin response to infection the test should be regarded not as evidence of infection in individual females but as a herd test, and the results interpreted on the basis of the proportion of positives and their incidence in relation to the time at which animals could first have been exposed to infection. If clear-cut and easily interpretable results are to be obtained, samples should be taken from heifers first exposed to infection between four and ten weeks previously. There is little point in taking specimens for testing at times earlier than four weeks after primary exposure.

If mucus specimens can be taken for testing at the time in the acute period of infection when agglutinins are most commonly present, there is usually no difficulty in the interpretation of the results because a high proportion of positive reactions occurs. But later in the course of the disease this may not be the case and the results may be difficult to interpret. It is generally agreed that any positive reactions should be regarded as evidence of infection, particularly where the history and symptoms are indicative of genital vibriosis. Some aid may be obtained by resampling, where only indefinite or suspicious reactions occur, or where only a small number of positive reactions are found. Provided that a suitable antigen is used, it is unlikely that false positives will result. Common antigens exist between *V. fetus* (*venerealis*) and *V. fetus* (*intestinalis*) but reactions to the latter organism will be found only on isolated occasions and usually in association with abortion. Antigenic differences do, however, exist between strains of *V. fetus* and, because recent research on antigenic structure is somewhat contradictory through differences in technique and methods used in analysis, it is necessary, until details have become clarified, particularly in order to avoid false negative results, to compare those obtained by testing mucus against several antigens, including one or more made from *V. fetus* (*intestinalis*).

The results of the test are categorized as positive, suspicious, or negative.

The bull

An accurate diagnosis of infection in bulls may be made by isolation of *V. fetus* from semen or preputial washings, from vaginal or uterine material taken from heifers to which they have been mated, or after finding positive mucus agglutination results in such heifers, or by a combination of these methods.

DIRECT CULTURAL EXAMINATION

Semen or preputial material should be collected with the minimum of contamination but, no matter what method is employed, both, and particularly preputial washings, tend to be contaminated to some degree. Semen collected with an artificial vagina as for insemination purposes is often suitable for cultural examination.

Table 13.– Isolation of *vibrio* from semen and preputial fluids of bulls in A.I. studs

Stud	Material collected	Number cultured	Number positive	Percentage positive
A	Ejaculates of semen	1 543	303	19.8
	Preputial fluids	130	16	12.3
B	Ejaculates of semen	508	88	17.3

Table 14. – Rate of isolation of *vibrio* from semen collected by means of the artificial vagina from known infected bulls

Stud	Number of specimens cultured	Number of specimens positive	Percentage
A	675	256	37.9
B	216	54	25.0

Some success has been obtained in the use of such material with a semi-solid thiol medium with 0.05 percent glutathione. The results which have been obtained by this method are set out in Tables 13 and 14.

Success has also been obtained in cultivating the organism from heavily contaminated semen and preputial washings by the use of

ordinary blood-agar plates containing 0.01 percent alkylaryl-sulphonate (Santomerse) and 0.5 percent aesculin, by the use of medium with 2 percent brilliant green (Florent and Vandeplassche, 1956), brilliant green and polymyxin (Power, 1958) and medium containing 10 percent ox-bile (Frank, personal communication). But complete reliance cannot as yet be placed upon a single examination (Akkermens. Terpstra and van Waveren, 1958; Morgan *et al.*, 1958).

Test-mating of heifers

Because of the difficulties often encountered in the isolation of *V. fetus* from semen and preputial specimens and which are largely associated with small numbers of organisms and the rapid overgrowth of contaminants, attempts have been made to evolve methods of isolation by making use of animal inoculation. Various methods have been tried, using among other animals pregnant and non-pregnant guinea pigs and hamsters. The results on the whole have been unsatisfactory and unreliable, mainly because no species has been found to be regularly susceptible to infection. Recourse must often be made, therefore, to attempts to recover the organisms from maiden heifers to which suspected bulls have been mated.

There is some doubt as to the number of heifers which must be used for this purpose. Tests in which cultural examinations are made of vaginal discharge or cervical and endometrial tissue obtained by means of a biopsy curette seem to require fewer animals than those involving the detection of mucus agglutinins developed after infection. It does appear that the great majority of previously unexposed females are highly susceptible to infection, but that there is some variation in the time and degree of response to infection in the development of mucus agglutinins.

Experience in Denmark, covering the testing of 638 bulls by biopsy methods (Adler, 1956), indicate that reliable results can be obtained by the use of one heifer for each bull. The test is carried out on maiden heifers 12-18 months old, shown previously to be negative to the mucus-agglutination test. Biopsy specimens are obtained from the cervix and posterior part of the uterus by means of a Folmer Nielsen curette, three to four days before the heifer is to be used. The corpus luteum is expressed at the same time and, when oestrus occurs after three to four days, sampling is repeated. The biopsy specimens

are used to inoculate media for culture as described above. Semi-solid media have been used but there is no reason to believe that other types of media will be less satisfactory. These specimens must be negative or the animal is discarded. The animal is then inseminated with semen to which preputial washings have been added. It has been determined experimentally that the chances of infecting heifers by this method are increased if preputial material is added. Two or three specimens of semen are collected with an artificial vagina and then the prepuce is irrigated with a small quantity of normal saline and the washings collected. The samples are then centrifuged for 15 minutes at 3,000 r.p.m., the deposits mixed, and 2 millilitres deposited in the heifer's cervical canal by means of an insemination pipette. Two to four days after insemination and at intervals of three to four days thereafter, further biopsy specimens are collected as above and inoculated into suitable media. Negative cultures from four samples are regarded as evidence that infection has not occurred and it is considered that the heifer may be used again to test specimens from another bull. Biopsy specimens are taken once more, simultaneously with enucleation of the corpus luteum, and immediately before renewed insemination. A negative diagnosis in a bull is thus based on six negative biopsies within a period of about three weeks.

To obtain specimens of material from the endometrium and cervix, a sterilized Folmer Nielsen uterine biopsy curette, 8 millimetres in diameter, contained within a narrow sterilized metal tube, is introduced into the vagina and passed through the cervix by rectal manipulation. After a portion of endometrium has been scraped off, the curette is withdrawn into the tube and removed. A second sample is taken from the cervix in a similar fashion. The specimens can thus be taken and sent to the laboratory with the minimum risk of contamination. Specimens of mucus taken simultaneously from the anterior vagina proved less reliable for diagnostic purposes, either because no *V. fetus* could be recovered although present in biopsy samples from the uterus, or because they were heavily contaminated with vaginal commensals.

Results from 89 heifers used for testing in this fashion show that, in 60 percent, diagnosis could be made on the basis of specimens withdrawn within five days, and in another 35 percent within nine days. In five heifers the diagnosis was not made until the tenth and

eleventh days (Table 15). It has been demonstrated by small-scale experiments that the reliability of the test is not increased by the use of two or three heifers instead of one, all animals becoming infected (Table 16).

Table 15. – Interval after exposure at which first positive biopsy was made in 89 heifers

	Days after infection				Diagnosis		Days after infection				Diagnosis		Days after infection				Diagnosis	
	2	3	4	5	total	percent positive	6	7	8	9	total	percent positive	10	11	12	13	total	percent positive
Number of heifers	9	11	27	0	53	59.6	10	11	3	7	31	34.8	3	2	0	0	5	5.6

Table 16. – Recovery of *V. fetus* by biopsy after exposure of more than one heifer

Bull	Number of heifers	Number of *Vibrio fetus*-infected heifers
N	2	2
N	2	2
N	3	3
N	3	3
H	2	2
T	3	3
T	3	3

Other workers have obtained better results from cultural examination of vaginal mucus taken in the acute phase of infection during the period from 10 to 30 days after exposure, the interval from 17 to 20 days being the optimum. In all cases, however, precautions must be taken to avoid contamination of the specimen.

Information concerning the use of the mucus-agglutination test in heifers for determining the presence of infection in bulls which have been mated to them is much less precise, particularly with regard to the number of heifers which must be used for each test. There is general agreement that it must be not less than six. They must

give negative mucus agglutination results before the test is begun. Specimens should be taken at intervals after exposure to infection, beginning at five weeks and repeated at monthly intervals thereafter. Presumably, a positive reaction in one heifer can be accepted as evidence of infection. This method in its present form is clearly less accurate, more cumbersome, time-consuming and expensive than that described above.

Technical methods employed

Methods of obtaining specimens of mucus for bacteriological examination

Several methods have been devised for obtaining uncontaminated specimens of mucus. In all, the vulva and surrounding perineum are cleaned and a sampling tube is inserted into the vagina and mucus drawn into it.

The simplest method is that in which the mucus is collected by means of a glass tube about 50 centimetres in length and 1 centimetre in diameter. This pipette has a slight bend about 10 centimetres from one end, which is lightly plugged with cotton wool. The other end is tightly plugged with cotton wool to act as a stop for the mucus. Before use the pipettes are wrapped in grease-proof paper and sterilized by autoclaving.

A piece of pressure rubber tubing about 50 centimetres long is attached to the straight end of the pipette, the plug at the bent end is removed, and this end is passed into the vagina as far as the cervix. Then, by sucking on the free end of the rubber tubing and moving the pipette backward and forward in the vagina, a portion of mucus is loosened and drawn into the pipette. The suction is maintained as the pipette is withdrawn from the vagina, a small sterile rubber stopper is inserted into the pipette, and a label with the identity of the animal is attached. In the laboratory, the mucus is forced from the pipette into a test tube by applying pressure on the cotton wool plug at the straight end of the pipette with a length of flexible wire.

Alternatively, the mucus may be aspirated by means of a sterile glass tube 10 millimetres $\times$ 36 centimetres, with a side opening at one end and a 2-ounce rubber bulb at the other. The mucus so obtained is deposited in a sterile container for transport to the laboratory.

Specimens may also be obtained by means of an instrument consisting of a telescopic tube, the large outer tube being 0.635 centimetre (0.25 inch) in outside diameter, with a 0.09-centimetre (0.035-inch) thick wall, and 45.7 centimetres (18 inches) long, and the smaller inner tube approximately 0.455 centimetre (0.179 inch) outside diameter, with an inside diameter of approximately 0.32 centimetre (0.125 inch) and 59 centimetres (22 inches) long. Inside is a steel wire 0.1 centimetre (0.039 inch) in diameter, by 58.4 centimetres (23 inches) long, with a loop made at one end into which can be placed a small piece of cotton wool to act as a plunger for forcing the contents from the small tube. The wire plunger is inserted to such a depth that it allows the cotton wool to remain in the centre of the small tube. A rubber tube, 0.32 centimetre (0.125 inch) inside diameter by 33 centimetres (13 inches) long, is fitted with a mouthpiece, placed over the protruding wire, and connected to the small metal tube. A rubber diaphragm cut from a surgical glove and about 1.9 centimetres (0.75 inch) square is stretched over the end of the large tube and secured with stout thread in a groove cut round the tube, 0.32 centimetre (0.125 inch) from one end. The small tube is inserted into the large tube, and the mouthpiece and rubber diaphragm enclosed in cotton wool before wrapping the apparatus in paper for autoclaving.

To obtain a specimen, one of the operator's hands is inserted into the rectum to locate the area which is to be sampled and the assembled apparatus is inserted into the vagina to within about 5 centimetres (2 inches) of the cervix. An assistant then pushes the small tube forward about 5 centimetres (2 inches), so breaking the rubber diaphragm on the large tube. The end of the small tube, when exposed for obtaining a specimen, is thus free from contaminants from other parts of the tract. Suction is now applied through the mouthpiece to draw the mucus into the tube. To withdraw the instrument, a vacuum is maintained with the mouth, and the small tube is withdrawn about halfway through the large tube. The apparatus is then removed from the vagina. Before the small tube is removed, the larger tube is sterilized by flaming. The mucus can then be forced out from the small tube into a suitable sterile container by means of the cotton wool swab.

Method of obtaining specimens of mucus for agglutination testing

Mucus may be collected for testing either by means of glass pipettes or special small gauze tampons. Tampons are most convenient for the collection of large numbers of specimens under some conditions. Pipettes are used as in the first method described above for the collection of mucus for bacteriological examination, except that they are not stoppered afterwards but the cotton wool plug is re-inserted. A separate pipette is used for each animal.

They are sent to the laboratory with the mucus *in situ*. It is then removed by applying pressure at the straight end of the pipette with a length of flexible wire.

Tampons are employed as follows:

The vulva and adjacent parts are washed with soap and water. The lips of the vulva are parted. A wide tube containing the tampon and a loose plunger is inserted into the vagina. The tampon is pushed out of the containing tube by means of the plunger and placed as far forward as possible in the vagina. The plunger and tube are withdrawn, the thumb being placed over the end of the tube so that air does not enter the vagina and cause it to balloon. The free end of the string attached to the tampon and which protrudes should be placed inside the vulva to prevent its becoming soiled by faeces. The tampon should be left *in situ* for at least 20 minutes in order to obtain an adequate sample; it is then removed by means of the string, the vulva first having been washed. The tampon and string are then placed in a 1-ounce, wide-mouthed, screw-top jar, which has been previously weighed, and each sample is marked for future identification.

Tampons can also be inserted through discardable stiff paper cylinders by means of a wooden plunger, a separate tube being used for each animal, thus avoiding the risk of mechanically transmitting infection.

Technique of the mucus-agglutination test

Where mucus has been obtained by means of pipettes, each sample is diluted with physiological saline in the proportion of one part of mucus to approximately four parts of saline, and then ground to a

homogenous suspension in a Griffith tube. The test involves the use of agar as a permeable membrane for the extraction from the vaginal mucus of the antibodies with phenol-saline and the use of this extract in a tube-agglutination test. The extract is water-clear and for this reason does not complicate the reading of the test.

Two millilitres of this mucus-saline suspension are transferred to a test tube which is put in a water bath at 57°C. To this is added 2 millilitres of molten agar (3 percent "Davis" powdered agar: 0.5 percent sodium chloride) also at 57°C.

The mixture of mucus, saline and agar is shaken vigorously and poured into small, 30 millilitre screw-topped glass jars, about 6 centimetres in diameter, and allowed to stand. When set, in about half an hour, 2 millilitres of 0.5 percent phenol-saline is layered on. The lid, with a paper washer to prevent excess evaporation, is screwed on tightly and the jar is incubated for 18 hours at 37° C. to allow the extraction process to take place.

The water-clear supernatant fluid is used in a five-tube serial dilution-agglutination test.

Five tubes (50 millimetres × 8 millimetres) are set up in a rack. In the first stage, the first tube is left empty but 0.5 millilitre of 0.5 percent phenol-saline is put into each of the other four. In the second stage, 0.5 millilitre of the supernatant fluid is put into each of the first and second tubes. The contents of the second tube are mixed and 0.5 millilitre carried over into the third tube. The process is repeated until the fifth tube is reached, from which, after mixing, 0.5 millilitre is discarded. In the last stage, 0.5 millilitre of antigen suspension is added to each tube and mixed by shaking.

The tests are incubated at 37° C. for 18 hours and read by ordinary transmitted light against a dark background and recorded thus:

+ + + +	— water-clear
+ + +	— about 75 percent clear
++ +	— about 50 percent clear
+	— about 25 percent clear

Any agglutination at all is regarded as denoting suspicion of infection, but a positive report is not given unless there is at least 75 percent clearing in the first two tubes.

In a herd where the initial tests reveal only animals with definite but less than 75 percent agglutination in the first two tubes, further

specimens are tested, as such results are regarded as suspicious but not positive.

In a series of tests when " Davis " agar was not available and other agar was being used, several animals known not to be infected were tested and showed some degree of clearing in the first, and sometimes the second tube. Split sample tests proved that the agar in question was at fault, and on using " Davis " agar, which is now specified as essential for the test, these animals all gave negative results.

The test may be modified in its preliminary stages as follows. Approximately 0.5 millilitre of mucus is diluted and thoroughly mixed in a Griffith tube with 7.5 millilitres of normal saline. The diluted mucus is transferred to a centrifuge tube and left at room temperature for one hour. It is then centrifuged for ten minutes at 300 r.p.m. The supernatant fluid is removed and used as " serum " in the agglutination test.

Mucus obtained by means of tampons must be treated somewhat differently preparatory to testing. The weight of the mucus is determined, usually 1 to 3 grammes being obtained. It is then diluted, using formolized (0.3 percent) physiological saline (0.8 percent) solution. Since the final 4 to 5 millilitres of liquid in the tampon cannot be readily expressed, dilutions must be made on the basis of sample size to ensure that sufficient material is available for testing purposes. For example, a 1-gramme sample must be diluted to 1 : 12.5 by adding 11.5 millilitres of saline to the sample jar. The diluted material may then be used directly in the first agglutination tube where, after the addition of antigen, the resultant dilution will be 1 : 25. Smaller samples may be used but low dilutions cannot be used for the test. Larger samples (over 1.5 grammes) are diluted by adding 5.25 millilitres of saline for each gramme of vaginal secretion obtained. Diluted samples are held at refrigerator temperature until use, usually overnight. The agglutination test is then carried out in the ordinary way, using serial dilutions.

The method of interpreting the agglutination results described above is typical of that used in most laboratories. When parallel tests are carried out against several antigens, a combined interpretation must be made.

It is possible that, in the light of more recent work on the antigenic structure of *V. fetus*, the antigens used and the interpretation placed on results may be further modified. There is no doubt that,

if the test is to be standardized and put on a completely reliable basis, much more information is still necessary, and this can be obtained only by further extensive research.

Production and standardization of antigen for mucus-agglutination testing

The antigens used should be composed of a wide range of antigenic components which will cover, as far as possible, the antigenic strains responsible for the natural disease. To achieve this it may be necessary to use several differing antigens. It has been found that the best results are generally obtained from the use of an antigen prepared from a strain isolated from an outbreak involving infertility, as distinct from one where only abortions were occurring. There is reason to believe also that there may be some advantage in using a local strain.

Before they are selected for use as antigens, strains should be tested against a variety of known positive sera to ensure that they have a wide antigenic make-up. They must also be shown to have a suitable degree of sensitivity, and their behavior with the sera of normal uninfected animals must be ascertained. It is desirable that they should not be agglutinated by normal sera but at the same time should be as sensitive as possible. After adoption as antigens they must be tested regularly against standard sera.

A variety of types of media are used for the routine growth of antigen, chocolate-agar being probably most commonly employed, although solid thiol is sometimes used.

The methods of production and standardization described here are those used at the Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Weybridge, England; at the State Serum Institute in Rotterdam, the Netherlands; and in the Microbiology Department of the Department of Hygiene and Bacteriology of the Royal Veterinary and Agricultural College, Copenhagen, Denmark. They exemplify the methods used elsewhere, although differences of detail may exist at other laboratories.

Weybridge method

Stock cultures, two strains of which, Longsdon and 1980, are used for antigen production, are kept in peptone blood-broth and are subcultured every 14 to 21 days.

The antigen is grown on chocolate-agar. Roux flasks of this medium are seeded, each with 4 millilitres of a three-day broth culture and incubated in bins containing 50 flasks in an atmosphere of 10 percent carbon dioxide at 37° C. for five days. The flasks are laid in the bins with the inoculated sides downward.

The growth is washed off with 0.5 percent formol-saline, about 30 millilitres per flask, with the aid of glass beads. The washings from each flask are collected separately and examined microscopically for purity. They are then bulked by pouring through muslin to remove large agar particles and the glass beads, and centrifuged at 1,500 r.p.m. for five minutes to throw down smaller pieces of agar, red blood corpuscles and debris. The deposit is discarded and the bacterial suspension is centrifuged at 3,800 r.p.m. for one hour. The supernatant fluid is discarded and the deposit is re-suspended in 0.25 percent formol-saline. This process of high-speed centrifugation and re-suspension in 0.25 percent formol-saline is again repeated twice. The antigen is finally re-suspended at 20 times the concentration at which it is finally used, and stored at 40° C. for at least one week before standardization and use.

When the antigen suspension is to be used, it is diluted to an opacity of between Brown tubes 2 and 3. It is then further standardized by tests against a standard freeze-dried serum.

Stock cultures have also been freeze-dried, and the survival rates have been in the region of 0.5 percent, although in some cases viable organisms have been recovered from cultures freeze-dried over 15 months earlier.

Standardization with freeze-dried serum

A large volume of blood was obtained from an animal, the serum of which gave a high titre when tested against antigen 1980. This serum was sterilized by passing through a Seitz (EK) asbestos pad, freeze-dried and sealed under vacuum in 1-millilitre ampoules. It gave 50 percent agglutination at a titre of 1 : 840 with the batch of 1980 strain antigen which was in use at that time. Subsequent batches of 1980 antigen have been standardized to give a like degree of agglutination with this serum.

The procedure, which follows closely the technique developed for the standardization of *Br. abortus* antigen suspension, is as follows.

An ampoule of dried serum is opened and 1 millilitre of distilled water added. The reconstituted serum is made up to 40 millilitres in a measuring cylinder with 0.5 percent phenol-saline, the ampoule being rinsed out thoroughly. The diluted serum is used to prepare stock quantities of about 50 millilitres at dilutions of 1:280, 1:350, 1:420, 1:490 and 1:560 in 0.5 percent phenol-saline in bottles with ground glass stoppers. These stock solutions are kept in the refrigerator but discarded after three months if they have not been used.

If, for example, the concentrated antigen suspension has to be diluted 1:20 to bring its opacity to between Brown opacity tubes 2 and 3, then 20 millilitres are prepared at this dilution and further similar quantities at dilutions of 1:17 and 1:23 in 0.25 percent formol-saline.

Each dilution of serum is tested in quadruplicate against each dilution of antigen suspension, thus:

Twelve racks are prepared, each rack holding five agglutination tubes, i.e., one tube for each dilution of serum.

Into the first tube in each rack, 0.5 millilitre of serum at the 1:280 dilution is pipetted; into the second tube, 0.5 millilitre of serum at the 1:350 dilution, and so on to the fifth tube in each rack, in which 0.5 millilitre of serum at the 1:560 dilution is placed.

Next, into the tubes in the first four racks, 0.5 millilitre of the antigen suspension at the 1 : 17 dilution is placed, and into the tubes in the second and third lots of four racks, respectively, 0.5 millilitre of the antigen suspension at the 1:20 and 1:23 dilutions is pipetted.

This process doubles the dilution of the serum in each tube to 1:560, 1:700, 1:840, 1:980 and 1:1,120 for the series.

The serum and antigen are thoroughly mixed by shaking and the tubes incubated at 37° C. for 23 hours before being examined in the usual way by transmitted light against a dark background. A suitable reading-box is necessary. Standards to represent 0, 25, 50 and 75 percent agglutination at each dilution of antigen are incubated at the same time. These standards are prepared in the following manner: 2 millilitres of diluted antigen suspension, e.g., 1:20, are added to 2 millilitres of 5 percent phenol-saline. This is double-diluted antigen.

Four agglutination tubes are set up in a rack.

TUBE 1 = 0 percent agglutination 1 millilitre double-diluted antigen
TUBE 2 = 25 percent agglutination 0.75 millilitre double-diluted antigen + 1/4 millilitre phenol-saline
TUBE 3 = 50 percent agglutination 0.5 millilitre double-diluted antigen + 1/2 millilitre phenol-saline
TUBE 4 = 75 percent agglutination 0.25 millilitre double-diluted antigen + 3/4 millilitre phenol-saline

These opacity standards are spaced on a rack and the tube to be read is matched between them.

The dilution of antigen suspension which comes nearest to showing 50 percent agglutination at a titre of 1 : 840 may, for example, be 1:23, and so a further series of tests is set up with dilutions of antigen at 1:21, 1:23 and 1:25. The dilution 1:23 may again give a 50 percent reading at a titre of 1:840, in which case the concentrated antigen suspension must be diluted 1 : 23 for use.

A similar method of standardization is used for the Longsdon strain, using a standard serum which originally agglutinated this antigen at a titre of 1:960.

Dutch method

The strain used is stored in the dark at room temperature and maintained on blood-agar slopes.

Seed cultures are made into tubes of bactothiol (Difco) and incubated at 37° C. for four days. After purity tests, the growth zone from four tubes is suspended in 100 millilitres of broth. Three millilitres of this suspension are spread evenly over the surface of chocolate-agar in Roux flasks. These are then incubated upside down in 15 percent CO_2 at 37° C. for four days. After the growth has been examined for purity both macroscopically and microscopically, the superfluous condensation water is sucked off.

The growth is then washed off with 0.2 percent formol-saline with the aid of a metal rod to the end of which a gauze swab is attached. The bacterial suspension so obtained from several flasks is pooled and, after filtration through absorbent gauze, is centrifuged at 1,000 r.p.m. for one minute. The bacterial suspension, with the exception of the coarse sediment, is again collected and washed by centrifuging at 15,000 r.p.m. for five minutes, after which the supernatant fluid is

discarded and the sediment carefully shaken up with saline. This washing is repeated once, after which the remaining bacteria are suspended in 0.2 percent formol-saline and stored as a concentrated antigen at 4° C.

For use in the agglutination test, the antigen is diluted with 0.2 percent formol-saline to a density corresponding to MacFarland II tube with the aid of a photo-electric cell.

The diluted antigen is now tested for purity and sensitivity. For the latter purpose the results of agglutination tests are compared with those obtained in parallel tests against a known antigen. The following types of sera are used for these tests:

(*a*) a homologous rabbit antiserum (stored freeze-dried);

(*b*) a heterologous rabbit antiserum (stored freeze-dried);

(*c*) at least three sera from cows believed *not* to have been infected with *V. fetus*;

(*d*) if possible, positive bovine sera or vaginal mucus specimens.

The strain from which the antigen is prepared was isolated from an animal in a herd in which infertility due to *V. fetus* infection was endemic. It still agglutinates almost to the same titre with homologous antiserum prepared soon after the isolation of the strain and since stored in a lyophilized condition. It was agglutinated to a high titre by nine antisera prepared from *V. fetus* strains isolated from cows which had a history of vibrionic infertility and is agglutinated either to a low titre (1 : 20-1 : 40) or not at all by sera from normal cows. The antiserum of this strain agglutinated to a relatively low titre antigens prepared from three strains of *V. fetus* isolated from cases of vibrionic abortion in a herd with no record of vibrionic infertility.

The antigen can be stored in the dark at 4° C. and without alteration in quality for at least a year. Irregularities in the preparation or use of certain batches of medium may lead to significant rises in the sensitivity. For example, antigens were prepared from three different strains of *V. fetus* seed cultures, blood-broth, thiol and blood-agar being used. Those prepared with blood-broth appeared to show a considerable rise in the non-specific reaction with normal sera.

Danish method

Three antigenically different strains of *V. fetus* are used:

1. American strain.
2. Dutch strain.
3. Danish strain.

The cultures are preserved by freeze-drying.

Seed cultures are prepared by transferring freeze-dried culture suspended in a small quantity of broth on to the surface of semi-solid medium in Roux flasks.

The semi-solid medium consists of Neopeptone Difco 1 percent, beef extract 1 percent, yeast extract Vitamin 0.5 percent, sodium chloride 0.5 percent, agar 0.3 percent. The pH of the medium is adjusted to 8.0. The medium is enriched just prior to use by adding 5-10 percent haemolized ox-blood solution prepared by dissolving fresh ox-blood in cold, sterile distilled water (1 : 3) and storing for one month at 5° C.

The Roux flasks are incubated for three days at 37° C. in 10 percent carbon dioxide. One millilitre of the growth layer of the semi-solid medium is transferred to the surface of chocolate-agar in Roux flasks for the antigen production.

The chocolate-agar consists of Bacto-peptone 1 percent, beef extract 0.5 percent, yeast extract Vitamin 0.3 percent, sodium thioglycolate 0.75 percent, sodium chloride 0.5 percent, ox-blood (with added sodium citrate) 5 percent, agar 2 percent. The pH of the medium is adjusted to 7.8.

Before inoculation with the seed culture, sterile glass beads are poured into the Roux flasks and 1-2 millilitres of saline added in order to obtain a really humid surface. After inoculation, the Roux flasks are incubated in 10 percent carbon dioxide. After two days, the incubation is interrupted and the inoculum is spread over the entire surface of the chocolate-agar by gently shaking the glass beads in the Roux flasks.

After four days, a sufficient mass of growth is usually obtained, and the antigen is harvested by adding saline and shaking the Roux flasks in order to loosen the growth layer through the rolling of the glass beads. The antigen suspension is filtered through gauze and finally washed by centrifugation at 300 r.p.m. for 60 minutes and a

concentrated antigen suspension is prepared. From this stock suspension the antigen is diluted for use with saline to the extinction of 0.6 in a 2.5-centimetre (1 inch) cuvette at 6,000 Å in a Unicam spectrophotometer, then preserved by adding 0.3 percent formalin and stored at 5° C.

Method of obtaining preputial washings

Preputial washings are best made by means of a blunt-ended catheter, about 51 centimetres (20 inches) long, to which a flutter valve apparatus and a 200-millilitre bottle are attached by about 90 centimetres (3 feet) of rubber tubing. The preputial orifice is clipped, thoroughly washed with soap and water, and swabbed with surgical spirit. The sterilized catheter, with its attachments, which have also previously been sterilized, is inserted into the prepuce to the level of the *glans penis*. The preputial orifice is occluded by means of a short length of gauze bandage and about 100 millilitres of sterile normal saline or nutrient broth is run by gravity into the prepuce from the bottle. The prepuce is then massaged through the preputial wall in order to wash off material from the mucous membrane and finally the fluid is allowed to run back by gravity into the bottle, simply by lowering the latter. Relatively uncontaminated specimens can be obtained in this way. Larger volumes of fluid may be used, but a less concentrated sample is then obtained and the recovery of organisms may be more difficult.

V. PREPARATION OF MEDIA USED FOR THE ISOLATION OF *VIBRIO FETUS* AND FOR THE GROWTH OF ANTIGEN

A large variety of media have been used for the primary isolation of *V. fetus*. That most commonly employed is blood-agar as plates or slopes. Semi-solid thiol medium is also used.

Chocolate-agar is used most frequently for antigen production.

Media for primary isolation

Bovine blood-agar

Bovine blood-agar is made up of 10 percent defibrinated bovine blood in nutrient agar. Nutrient agar consists of 1 percent Bacto peptone (Difco), 0.5 percent NaCl, 0.5 percent Lemco beef extract and 1.5 percent powdered " Davis " agar, and is prepared in the following manner.

The Lemco is dissolved in a little tap water and, after adjusting the pH to 8 with normal NaOH, it is heated to boiling point. It is then filtered through folded filter paper. The other ingredients are dissolved in the remainder of the tap water, the Lemco solution is added and the pH is again adjusted to 8. This mixture is autoclaved at 20 pounds pressure for 15 minutes, filtered through Whatman acid-washed filter paper pulp, and the pH adjusted to 7.6 with normal HCl. Finally, the nutrient agar is autoclaved at 10 pounds for 15 minutes. It is kept as stock in flasks in 250-millimetre amounts.

The nutrient agar is melted in the steamer, allowed to cool in the waterbath at 56° C., 10 percent defibrinated bovine blood is added and the medium is poured into Petri dishes or, if slopes are required, into test tubes.

The blood should be taken from an animal which is known never to have been infected with *V. fetus* and which has never shown any positive blood or mucus agglutination reaction to this organism.

Growth in this medium may sometimes be stimulated by the addition of 0.3 percent thiol.

Modified plastridge medium

Bactothiol (Difco)	25 grammes
Glutathione	0.05 gramme
Agar	19 grammes
Distilled water pH 6.8	1 000 millilitres

This medium is dispensed in screw-capped tubes, is autoclaved at 20 pounds pressure for 15 minutes and, if not used immediately, stored in the refrigerator. Before use, the tubes are sloped and, after cooling, 1 millilitre of ordinary broth containing 10 percent of defibrinated guinea-pig blood is pipetted into each tube. It should be freshly prepared. It is most convenient for culturing mucus samples, a drop of mucus being placed in its liquid portion. Good results from the culture of semen on this medium will be obtained only if the specimen has been collected without gross contamination.

One of the main advantages of this medium is that *V. fetus* does not require a carbon dioxide atmosphere when cultivated on it. It may be modified to a semi-solid state by using only 0.4 percent of agar.

Martin broth [1]

This is a mixture of two liquids:

1. *Peptone solution A*

(*a*) Wash thoroughly under running water five pigs' stomachs. Remove the fat and mince finely.

(*b*) To minced tissue – 200 grammes, add:
Pure HCl – 10 millilitres,
Water (at 50°C.) – 1,000 millilitres.

(*c*) Maintain the mixture at 50°C. in a water bath for 24 hours.

(*d*) Autoclave for 30 minutes at 100°C.

(*e*) Filter through absorbent cotton wool.

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[1] Sartory, A., R. Sartory and J. Meyer. 1950. *Microbiologie pratique*, Paris, Librairie Maloine.

(*f*) While still warm, adjust the pH of the filtrate to 7.2 with NaOH N/10.

(*g*) Filter.

(*h*) Dispense in glass containers of 1,000 millilitres.

(*i*) Autoclave for 30 minutes at 115°C.

2. *Meat extract B*

(*a*) Mince beef or calves' flesh without fat.

(*b*) To minced meat – 500 grammes, add:
Water – 1,000 millilitres.
Allow the mixture to stand for 24 hours at room temperature.

(*c*) Press through linen.

(*d*) Add to the extract NaCl, 15 grammes.

Mix 500 millilitres of A with 500 millilitres of B. Heat the mixture to 70° C. Adjust the pH to 7.2. Autoclave for 15 minutes at 115°C. Filter through Chardin paper. Autoclave for 20 minutes at 110°C.

Media for maintenance of stock cultures

BOVINE BLOOD-AGAR

See page 42.

PEPTONE BROTH

The ingredients are 1 percent Bactopeptone, 0.5 percent NaCl and 0.5 percent Lemco. They are dissolved in tap water and the pH adjusted to 8. The broth is put in the autoclave and the pressure is raised to 20 pounds and lowered immediately. It is then filtered through folded filter paper (Whatman folded filter paper No. 14) and the pH lowered to 7.6. The broth medium is dispensed in test tubes in 9-millilitre amounts and autoclaved at 10 pounds for 15 minutes.

Media for antigen production

FOR PREPARATION OF SEED CULTURES

1. Bactothiol (Difco).
2. Semi-solid medium (see Danish method of antigen preparation, page 40).

For antigen production

Chocolate-agar

Nutrient agar is prepared in the same way as for bovine blood-agar (see page 42) and poured into Roux flasks (140 millilitres per flask) which are then sterilized in the autoclave at 10 pounds for half an hour. As the flasks come from the autoclave, 10 percent of defibrinated bovine blood is added, mixed well, and the agar allowed to set.

The medium used in Denmark for the preparation of antigen differs slightly from that described above (see Danish method of antigen preparation, page 40).

VI. TREATMENT AND MANAGEMENT OF ESTABLISHED *VIBRIO FETUS* INFECTION

Once *V. fetus* (*venerealis*) infection has been diagnosed in a herd, the initial measure is to separate the herd into two groups. The first consists of males and females which have not been exposed to infection. In many cases this group will consist only of maiden heifers and bulls which have never been used for breeding. Breeding can be continued immediately in this group without any therapeutic measures, provided that it remains a separate entity. This separation is necessary only with regard to breeding because the chances of transmission of infection, except by contact at service, are so remote. Unexposed females may be bred with unexposed bulls or may be artificially inseminated with semen from non-infected bulls. If the disease is to be controlled effectively, particularly where natural service is used, there must be no doubt that the animals in this group have not, in fact, been exposed to infection. Should there be any element of doubt with regard to individuals, they should not be included. The decision as to whether or not they have been exposed to infection can be made only on breeding history. The mucus-agglutination test cannot be effectively used.

The second group consists of all the remaining animals of the herd, male and female, which have been, or may have been, exposed to infection. This group again must be retained as a separate entity for breeding purposes. Within it, fertility will gradually return almost to normal as resistance to infection is acquired. This may occupy a period of three to six months or, in some cases, longer, and a small proportion of chronically infertile animals may remain. There is some evidence that the course of the disease may be shortened if infected bulls are eliminated and infected cows are inseminated artificially with semen from non-infected bulls. The effect of the disease can be limited to some extent also by treatment of infected females,

(Table 17), but so far as is at present known, there is no therapeutic method which will completely cure infection in all animals treated, and cured animals remain susceptible to re-infection (Melrose, Morgan and Stewart, 1959).

Table 17. – Fertility in heifers cured by antibiotic treatment

	Number	Number pregnant	Total inseminations	Inseminations per pregnancy
Heifers cured by treatment	27	27	43	1.6
Heifers remaining infected	27	17	92	5.4

Whether breeding is continued between infected bulls and cows or artificial insemination from non-infected bulls is used with the cows treated or untreated, some undetectable, female " carriers " of the disease may remain. As a result, this group must continue to be regarded as a potential source of infection. It is, therefore, unnecessary to kill infected bulls, particularly if they are valuable breeding animals, as they can be utilized on infected females and reasonably satisfactory fertility will be obtained. It must, however, be borne in mind that their presence is to some extent a potential source of danger to the healthy animals on the farm because of the possibility that, either through accident or neglect, they may be used for breeding with these animals. Successful management of outbreaks of vibriosis depends, where infected bulls are retained, on stockowners being made to appreciate that such bulls are potentially dangerous to the non-infected stock. Equally, they must appreciate that non-infected bulls must not be used with cows and heifers in the infected group.

The period required for the infected group to become completely free from the disease still remains to some extent uncertain. Infection can remain throughout and after at least one pregnancy but there is good evidence that it rarely remains after two normal gestation periods, provided that the sources of infection, i.e., infected bulls or infected semen, have been removed, and the process of cure is undoubtedly aided by treatment of infected cows and heifers with antibiotics. Under field conditions, however, it is often extremely difficult to devise means of applying effectively the bacteriological tests which are necessary in order to determine whether a herd is completely free from

infection. The mucus-agglutination test cannot be used and a completely reliable decision as to whether or not an animal is free from infection can be based only on bacteriological examination of mucus samples. Because undetected "carriers," even if present only in very small numbers, would re-initiate the disease, extreme care must be taken before deciding that a herd has become free from infection and unrestricted natural service with uninfected bulls is employed.

It is probably most satisfactory, where there are valuable infected bulls which it is undesirable to slaughter, to remove them from the herd, treat them, apply suitable tests to determine the efficiency of treatment, then return the cured animals for breeding with non-infected females, by natural service or, with both the above-described groups, by artificial insemination.

The simplest and most easily practicable method of dealing with the infection, and the one which is least susceptible to error, is to use artificial insemination from uninfected bulls on both the infected and uninfected groups, but very often this is not possible.

Earlier work has indicated that treatment of *V. fetus* infection in both males and females with antibiotics, particularly penicillin and streptomycin, may be effective. In addition, it is known that the organism is sensitive *in vitro* to the action of these drugs. The results of earlier therapeutic trials are, however, somewhat difficult to assess because of the conditions under which they had of necessity to be carried out and the consequent absence of satisfactory controls. It is known also that improvements can be obtained in the fertility of some bulls used for artificial insemination by the addition of penicillin, streptomycin and sulphanilamide to semen diluents. This result was demonstrated originally without reference to the effect of any particular pathogens. Later work indicates, however, that the main action of antibiotics in the improvement of fertility is probably through the control of *V. fetus* infection, although in some cases they may counteract the effect of other organisms.

Further controlled experiments on females have produced conflicting and inconclusive results, even where the same antibiotic has been used in a similar manner and dosage (Tables 18 and 19).

It appears, however, that penicillin, streptomycin and aureomycin may all produce cures in some animals, the latter two so far having given the most promising results. Whether systemic treatment, local intra-uterine treatment or a combination of the two are most

likely to be effective, cannot as yet be decided. It is possible that local treatment may be necessary, as there is evidence that the non-gravid bovine uterus presents a partial barrier to the passage of antibiotics and prevents the build-up of an effective concentration of

Table 18. – Effect of treatment of heifers infected with *Vibrio fetus*

Number of animals	Treatment	Route of administration	Number of animals remaining infected after treatment
6	6-5 mega units crystalline sodium penicillin, in 10 millilitres distilled water, daily for five days	Intramuscular	4
6	1 mega unit procaine penicillin suspended in 10 millilitres arachis oil with 4 % aluminium stearate on day of oestrus and following day	Intra-uterine	2
6	5 grammes streptomycin sulphate in 10 millilitres distilled water daily for five days	Intramuscular	1
6	1 gramme streptomycin sulphate in 10 millilitres arachis oil with 2 % aluminium stearate and 3 % cetyl stearyl alcohol on day of oestrus and following day	Intra-uterine	3
6	5 grammes aureomycin in 500 millilitres distilled water daily for five days	Intravenous	0
6	0.5 gramme aureomycin hydrochloride in 10 millilitres propylene glycol on day of oestrus and following day	Intra-uterine	0
6	5 grammes chloromycetin in 12.5 millilitres distilled water daily for five days	Intramuscular	6
6	12.5 millilitres chloromycetin 10 % in propylene glycol	Intra-uterine	6
6	One thiosemicarbazone pessary daily for five days	Intra-vaginally	5

them within the uterus. It is evident that complete reliance cannot as yet be placed on any method of treatment and that further research is necessary.

Apart from the question of the most suitable drug, its dosage and

its mode of use, it is necessary to decide whether more effective therapeutic action can be obtained by its administration at some particular phase of the oestrous cycle. This may be particularly important in relation to local intra-uterine administration. The way in which

Table 19. – Treatment of heifers with antibiotics

Number of animals	Treatment	Route of administration	Number of animals remaining infected after treatment
11	2 grammes streptomycin in 40 millilitres oil + 2.5 % aluminium monostearate	Intra-uterine	5
10	1 gramme streptomycin in 20 millilitres oil for one day	Intra-uterine	6
27	1 gramme streptomycin in 20 millilitres oil for two days	Intra-uterine	8
24	1 gramme streptomycin in 20 millilitres oil for three days	Intra-uterine	2
10	0.5 gramme streptomycin in 20 millilitres oil for two days	Intra-uterine	6
	0.5 gramme streptomycin in 20 millilitres oil for three days	Intra-uterine	4
8	0.5 gramme aureomycin in 20 millilitres oil for one day	Intra-uterine	8
4	0.5 gramme aureomycin in 20 millilitres oil for three days	Intra-uterine	2
5	5 milligrammes streptomycin per kilogramme body weight, daily for three days	Subcutaneous	5

the suspension of antibiotics is distributed within the uterus may also affect the results of treatment. In some experiments, the material has been distributed deliberately between the uterine body and cornua, but it has been suggested that no particular care is necessary in this regard and that once a suspension is deposited within the uterus in either the body or the horns, it becomes evenly distributed throughout the whole organ.

It is often convenient to carry out intra-uterine treatment on the

day after oestrus, although there is nothing to indicate whether this is therapeutically more effective.

While complete cure cannot be reliably expected as the result of any of the present methods of treatment, there is no doubt that antibiotic therapy will, in many cases, reduce the level of infection and, with the development of immunity, aid recovery and shorten the course of the disease, and that it is the most promising line of treatment to follow.

Extensions of work on the action of antibiotics on *V. fetus* in semen have also brought conflicting results. The effectiveness of such treatment appears to depend on several factors not necessarily associated with the initial level of infection of the semen. Among these are the semen dilution rate, the concentration of antibiotics, the time during which semen is exposed to them, and the temperature at which they are allowed to act. The concentration of streptomycin cannot be made very high because fertility becomes reduced, probably because of a direct toxic effect on spermatozoa. Semen cannot be held for long at temperatures much above 5° C., for fertility is lowered because of the directly deleterious effect of such temperatures on spermatozoa.

It is considered that, if semen is diluted at least 1 : 25 in yolk citrate containing 500 units of penicillin and 500 microgrammes of streptomycin per millilitre and held at 5° C. for at least six hours before use, infection is not transmitted (Tables 20 and 21). Where semen has had similar concentrations of penicillin and streptomycin added together

Table 20. – Effect of treatment of semen diluted 1 : 25 with yolk citrate containing 500 units of penicillin and 500 microgrammes streptomycin per millilitre and held at 5° C. for at least six hours, on recovery of *Vibrio fetus* by culture

	Result of culture before dilution	Result of culture after dilution	
		total cultures	number +ve
Semen without antibiotics	– ve	14	1
	+ ve	18	9
Treated semen	– ve	271	0
	+ ve	201	0

Table 21. – Effect of treatment of semen diluted 1:4 or 1:25 with yolk citrate containing 500 units of penicillin and 500 microgrammes streptomycin per millilitre and held at 5°C. for at least six hours, on transmission of *Vibrio fetus* to maiden heifers

	Dilution rate	Number of heifers	Number infected
Semen without antibiotics	1 : 4	30	26
	1 : 25	29	4
Treated semen	1 : 25	94	0

Table 22. – Effect on semen of addition of antibiotics to the diluent

Number of animals	Method of treatment of semen	Number of animals infected with *Vibrio fetus*
12	Naturally infected. Diluted 1 : 6 with egg yolk citrate stored at 4°C.	12
23	Naturally infected. Diluted 1 : 6 with egg yolk citrate, 500 units crystalline sodium penicillin, 500 microgrammes streptomycin calcium chloride per millilitre and 0.3 % sulphanilamide added. Held at room temperature for two hours, then stored at 4°C.	5
12	Artificially infected by addition, after dilution 1 : 6 with egg yolk citrate of centrifuged deposit of three-day-old broth cultures of *V. fetus*	11
6	Diluted and artificially infected as above. 500 units crystalline sodium penicillin, 500 microgrammes streptomycin calcium chloride per millilitre and 0.3 % sulphanilamide added. Held at room temperature for two hours, then stored at 4°C.	3
6	Treated as above but with double the concentration of antibiotics	2

with 0.3 percent sulphanilamide, diluted 1 : 6 and held at room temperature for two hours before being stored at 4°C., a substantial proportion of animals became infected both with naturally and artificially infected semen. The use of double the quantity of antibiotics did not materially affect the results (Table 22). The incidence of infection was less than in untreated controls but the fertility was low (Table 23).

Table 23. – Fertility in heifers inseminated with infected semen to which antibiotics had been added

		Number	Number pregnant	Total inseminations	Inseminations per conception
Naturally infected semen + antibiotics	Infected animals	17	15	92	6.1
	Non-infected animals	18	18	34	1.9
Artificially infected semen + antibiotics	Infected animals	15	11	72	6.5
	Non-infected animals	9	9	20	2.2

These findings have been confirmed in experiments where semen diluted with egg yolk citrate at dilutions of 1:5 and 1:50 and containing 500 microgrammes of streptomycin per millilitre was used, but in experiments in which semen was diluted with reconstituted skim milk diluent containing 500 microgrammes per millilitre infection was not transmitted. There is no doubt that antibiotic treatment, and in particular treatment with streptomycin, materially reduces the risk of transmission of infection (McEntee, Gilman, Hughes, Wagner and Dunn, 1959). The organism does, however, survive on some occasions both in semen stored at –79° (Report, 1954) and at 5°C. In view of the importance of artificial insemination, both as a means of dissemination of infection, and as a method of control of the disease, it is urgently necessary to develop some completely reliable methods of suppressing infection in semen.

In the bull, promising results have been obtained, particularly following local antibiotic or antiseptic treatment of the penis, prepuce and terminal portion of the urethra. The response to a wide variety of treatments has, in one case, led to the view that infection in the male can be readily dealt with, provided that local treatment of the exteriorized penis and prepuce is carried out under epidural anaesthesia. Particularly favorable results have also followed the local use of acridine derivatives (Tables 24 and 25). Results were assessed either on the basis of mucus-agglutination tests on heifers served after

Table 24. – Treatment of bulls with antibiotics [1]

Drugs used	Number of bulls treated	Number cured	Number not cured
Terramycin, 500 microgrammes	2	1	1
Streptomycin, 250 microgrammes Penicillin, 500,000 units Streptomycin, 500 microgrammes	3	3	0
Streptomycin, 550 microgrammes Penicillin, 1,000,000 units Chloromycetin, 500 microgrammes	4	4	0

[1] All drugs were dissolved in distilled water and mixed with polyethylene glycol. 20 millilitres were injected into the urethra as liquid and the remainder massaged into the penile and preputial mucosa for 12-15 minutes.

Table 25. – Treatment of bulls with antiseptics [1]

Number of bulls treated	Number cured	Number not cured
19	18	1

[1] The penile and preputial membranes were washed in warm trypoflavin solution for 10 minutes. " Bovaflavin " ointment (Hoechst) was then massaged into them for 10 minutes and 10 millilitres trypoflavin was injected into the urethra.

treatment or negative cultural *post-mortem* examination of the uteri of heifers served after treatment.

Thirty bulls treated later with trypaflavine and bovoflavine and 85 with streptomycin and terramycin were cured, care being taken to examine suitable material bacteriologically at intervals afterward.

These results are confirmed by other workers who have obtained complete cures in 14 bulls which were treated locally under epidural anaesthesia on the first day with 750.000 units of procaine benzylpenicillin, 250,000 units of potassium benzylpenicillin and 700,000 milligrammes of dihydrostreptomycin sulphate in 50 millilitres of oil, diluted with 50 millilitres of distilled water and shaken into an emulsion. Twenty millilitres of the emulsion were injected into the urethra and the remainder massaged into the penile and preputial mucous membranes and round the preputial orifice. On each of four successive

days, without epidural anaesthesia, 50 millilitres of a similar emulsion were injected into the prepuce and distributed throughout it by massage with the preputial orifice closed (Edgson and Scarnell, 1955). All of seven bulls treated without epidural anaesthesia with 2 grammes of streptomycin and 1 mega unit of penicillin in oil injected into the prepuce daily for three days recovered, as did all of six treated with the same drugs incorporated in an ointment (Melrose, Morgan, Stewart and Thomson, 1957). But in another case one of 14 animals treated remained uncured, although cured by subsequent treatment (Lawson and MacKinnon, Personal communication).

There is every reason to regard treatment of infected bulls optimistically but, in view of results obtained in Denmark, where a large number of relapses or re-infections have occured in treated animals (Adler and Landegaard, 1958) (Table 26), it does seem essential that treatment should be followed by adequate diagnostic tests.

Table 26. – Results of treatment of bulls with antibiotics assessed at intervals after treatment

Number of treatments	Status of bulls at intervals after treatment		
	1 month	6 months	12 months
214	167 (85 %) not infected	79 (72 %) not infected	41 (73 %) not infected
	29 (15 %) infected	30 (28 %) infected	15 (27 %) infected
	18 not tested	58 not tested	23 not tested

It is essential also that treated animals should be rehoused in premises which are free from infection and should not come in contact with other infected bulls either directly or indirectly. There seems little doubt that there are special risks attached to the spread of infection among bulls at artificial insemination centres, although the epidemiological factors involved are not understood.

Infection with *V. fetus* (*intestinalis*) does not pose difficult problems of treatment because of its sporadic nature and the fact that the organism is not spread by the bull. There is in fact little information concerning its treatment, but it seems likely that recovery should be at least aided by intra-uterine and systemic treatment with streptomycin and penicillin in ordinary therapeutic doses.

VII. PREVENTION AND CONTROL OF GENITAL VIBRIOSIS

The general control of *V. fetus* (*venerealis*) infection, as with that of most other infectious diseases, depends primarily on the detection of centres of dissemination of infection by accurate diagnostic measures, the elimination of such centres and the prevention of transmission of infection during the process. In the case of vibriosis, matters are simplified to some extent through the disease being transmitted only venereally, except on rare occasions. Whether control should be exercised by compulsory measures involving notification of the disease, or through voluntary schemes, or a combination of both, is a matter which can be decided only by local circumstances. In some countries, compulsory notification of *V. fetus* infection and control measures based on this have been very successful and have markedly reduced the incidence of the disease.

The two main *foci* from which the disease may be spread are the large pedigree breeding herds from which bulls are purchased, and artificial insemination centres. Whether or not the measures applied are voluntary or compulsory, regular mucus-agglutination testing of cows in breeding herds, so that such herds can be certified as being free from infection, is probably the best method which can be applied at present to reduce the spread of the disease from these herds, although its application may not always be practicable.

Because of the variation in the way in which individual animals produce mucus agglutinins as the result of infection, the results of the test can only be assessed on a herd and not on an individual animal basis. But once it is satisfactorily established that a herd contains no reactors to a suitably comprehensive and accurate test, all classes of stock in it can be accepted as free from infection, provided that they are not in contact with infected cattle. Where voluntary schemes are used, their establishment and success depend, to a very large extent,,

on education of stockowners as to the method by which the disease is spread and the results it produces.

Because infection can be disseminated on a very wide scale from artificial insemination centres, there is little to be said in favor of not using compulsory measures to eliminate the disease from them if it exists, or to prevent its entrance and ensure that the only bulls used are those which are free from infection. Infected animals can be detected and eliminated or treated, following the use of the diagnostic measures already detailed. The success of any treatment employed can be assessed in the same way. Absence of infection from bulls brought on to centres can be ensured if they have never been used previously for breeding, or if they have been used only with maiden heifers, or if they are from herds which are free from infection, or, in the case of other older bulls, if suitable diagnostic tests have first been applied to them and found negative. Once non-infected bulls are at centres they must be maintained apart from infected or untested stock. Particular risk is attached to the common practice of sending out bulls to farms and using them for natural service until they are proven. There is also a substantial risk of the infection being spread from bull to bull at artificial insemination centres.

In some countries, the incidence of *V. fetus* is so high among bulls at artificial insemination centres that the elimination of infected animals is impossible. In such cases where infected animals cannot be treated, it is possible to control the disease up to a point by the use of antibiotics in semen diluents. It must, however, be emphasized that at present no antibiotic or combination of antibiotics used by any known technique will completely destroy *V. fetus* in semen on every occasion. Even should a consistently efficient technique be evolved, the risk of mistakes arising under the conditions which obtain at many artificial insemination centres is so great that it seems unwarranted to place complete confidence in the use of antibiotics to control infection of semen. While the level of transmission of infection may be reduced by this method, and fertility be brought back almost to normal, transmission of the disease even to a slight degree can start new *foci* of infection because of the combined use of artificial and natural breeding in many herds.

Individual herds may be maintained free from infection by the use of the same methods as have been outlined above. The only bulls which should be purchased for use in such herds are those from

herds free from infection, those which have never previously been used for breeding or have been used only with maiden heifers, or those to which suitable diagnostic tests have been applied and found negative. Only female animals in analogous categories should be purchased, i.e., maiden heifers or cows from herds known to be free from the disease. There is no diagnostic test which can be applied to individual mature cows.

The same method of prophylaxis as is suitable for individual herds may be applied to prevent the disease entering countries in which it does not exist.

Control of the disease depends to a great extent on the availability of artificial insemination from uninfected bulls and the accessibility of efficient laboratory diagnostic services. In many countries these are not available and may take some years to develop. It may therefore be that in such areas some control might be obtained by the use of an effective vaccine, although none exists at present.

Infection with *V. fetus* (*intestinalis*) is, so far as is known, a subsidiary problem because of its sporadic occurrence and the fact that it is not spread venereally. Individual cases can therefore be dealt with as they occur. When more is known of its mode of pathogenesis and its association with the disease in sheep, measures for its control may be designed.

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